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FORMALIN-INACTIVATED VENEZUELAN EQUINE
ENCEPHALOMYELITIS (TRINIDAD STRAIN)
VACCINE PRODUCED IN ROLLING-BOTTLE
CULTURES OF CHICKEN EMBRYO CELLS

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Formalin-inactivated Venezuelan Equine Encephalomyelitis (Trinidad Strain) Vaccine Produced in Rolling-Bottle Cultures of Chicken Embryo Cells

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Formalin-inactivated Venezuelan equine encephalomyelitis vaccine was prepared from virus grown in rolling-bottle cultures of chicken embryo cells. Trinidad strain virus was propagated in these cultures with a maintenance medium consisting of serum-free medium 199 containing 0.25% human serum albumin (USP) and antibiotics. Manipulation of multiplicity of inoculum (0.06 to 0.00006) and maintenance medium volume (100 to 300 ml) resulted in high-titered virus yields and only moderate cell destruction when fluids from infected cultures were harvested at 18 to 24 hr. The virus was inactivated at 37 C by 0.05% Formalin within 8 to 10 hr and with 0.1% Formalin within 6 to 8 hr. Single dose antigen extinction tests in mice performed with 30 small-scale vaccine lots showed excellent potency at either Formalin concentration with inactivation periods ranging from 24 to 96 hr.

The need for an inactivated Venezuelan equine encephalomyelitis (VEE) vaccine is manifold. The currently employed attenuated (TC-83) VEE vaccine (4, 9), although highly protective, causes mild to moderate systemic reactions in 20 to 40% of human vaccinees (1; P. J. Bartelloni, *personal communication*). Based on our experience with inactivated Western and Eastern equine encephalomyelitis (WEE, EEE) vaccines in man (2, 3), one can anticipate that a VEE vaccine, similarly prepared, would be of equally low reactogenicity. Moreover, a VEE vaccine could be incorporated with existing inactivated WEE (2) and EEE (10) vaccines to achieve a trivalent product. This combined immunogen would be of logistical advantage for use in at-risk laboratory personnel or field personnel as well as for *Equidae*, the significant amplifying hosts for VEE.

The need for an inactivated VEE vaccine has been given greater impetus due to the presence of epizootic VEE in the United States and the above-cited observations. This paper describes the development of such a vaccine.

MATERIALS AND METHODS

Virus. Trinidad strain VEE virus had a history of

one guinea pig passage followed by 13 passages in embryonated hens' eggs (12). The 13th egg passage material was grown in rolling-bottle cultures of chicken embryo cells (CEC) to produce seed virus for vaccine production. A 20% suspension of the 13th passage chicken embryo preparation was used as challenge virus for potency assays.

Virus titration. Three-week-old or 1- to 3-day-old white mice (CD-1 strain of Charles River Mouse Farms, Wilmington, Mass.) were inoculated intracerebrally (ic) with 0.03 ml of log₁₀ dilutions of virus in cold phosphate-buffered saline (pH 7.2) containing 1% normal rabbit serum. Six mice were used for each dilution. After a 10-day observation period, titration end points were determined by the method of Reed and Muench (14) and are expressed as median lethal doses (LD₅₀) per ml.

Cell cultures. Rolling-bottle (280 mm; Bellco Glass, Inc., Vineland, N.J.) cultures of CEC were prepared as previously described (5). Cultures were incubated at 35 C until confluent cell sheets were obtained (18 to 24 hr). Growth medium was replaced with a maintenance medium (MM) consisting of serum-free medium 199 containing 0.25% human serum albumin (USP) plus 100 μ g each of neomycin (USP) and streptomycin (USP) per ml, and the cultures were incubated an additional 20 to 24 hr at 35 C.

Inoculation of cell cultures. MM was decanted from cultures, and residual fluids were drained from the cell sheets. The infecting virus in 10 ml of MM

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was added to each culture and adsorbed for 1 hr at 35 C with the roller apparatus set at 0.7 rev/min. To study the effect of multiplicity of inoculum (MOI), cultures were inoculated with VEE seed virus at MOI of 0.06 to 0.00006 (i.e., 0.96 to 0.00006 mouse LD₅₀/per cell) and maintained with 200 ml of MM. Culture fluid samples were removed at regular intervals postinoculation and were assayed as described. In all further studies herein, an MOI of 0.006 was employed; this was a 10^{-4} dilution of the CEC virus seed material.

Vaccine preparation. Prior to inactivation, fluids from infected cultures were centrifuged at $1,000 \times g$ for 30 min at 4 C and were then passed through a membrane filter (0.45- μ m pore size; Millipore Corp., Bedford, Mass.) to remove cellular debris. Within 1 hr of filtration, Formalin (formaldehyde, 37% assay) was added to final concentrations of 1:2,000 (0.05%) or 1:1,000 (0.1%). After thorough mixing, the fluids in closed vessels were placed at 37 C in a reciprocating water bath (New Brunswick Scientific Co., Inc.) set at 150 rev/min. Fluids were thus kept in gentle, but constant movement throughout the inactivation periods of 24 to 96 hr. At the end of the desired period of inactivation, the material was held in closed vessels at 4 C for 15 days during which time the vessels were shaken at least twice daily.

Assay of vaccines. Potency was determined by using a one-dose antigen extinction assay in 3-week-old, CD-1 white mice. Groups of 10 mice were inoculated intraperitoneally (ip) on day 0 with 0.3 ml of fivefold dilutions of vaccine. Fourteen days later the mice were challenged ip with 10^3 to 10^4 mouse ip LD₅₀ of VEE virus. Animals were observed for 14 days; titration end points and median effective dose (ED₅₀) values were determined as previously described (6).

Tests for safety, toxicity, and sterility. Representative lots of vaccine were tested in accordance with current U.S. government regulations (11). Testing was carried out as previously described (10).

RESULTS

Virus replication. Since similar virus yields were obtained at 20 and 24 hr postinoculation with all MOI tested (Table 1), a MOI of 0.006 was arbitrarily selected for use in subsequent experiments. This represented 10.0 ml of a 10^{-4} dilution of seed virus having a titer of 10^4 mouse LD₅₀/ml.

Effect of volume of maintenance me-

TABLE 1. Effect of multiplicity of inoculum (MOI) on propagation of VEE virus in roller-bottle chicken embryo cell cultures

Hr post-inoculation	Log ₁₀ LD ₅₀ /ml by MOI of			
	0.06	0.006	0.0006	0.00006
20	8.5	8.9	9.0	8.5
24	8.7	9.3	8.5	9.0

dium. To determine the effect of MM volume on virus yield, replicate cultures were infected at a MOI of 0.006 and maintained with 100 to 300 ml of MM. Shown in Table 2 are the results of titrations performed with fluids removed from these cultures at regular intervals. Although adequate virus titers were achieved with all MM volumes between 12 and 24 hr postinoculation, substantially fewer cytopathic effects occurred in those cultures maintained with 300 ml of MM. In general, cell destruction was moderate at 12 to 18 hr, but increased greatly by 24 hr postinoculation.

Virus inactivation. Samples were taken at regular intervals during the period of inactivation and titrated immediately in 1- to 3-day-old mice. Control virus harvests subjected only to 37 C for similar periods were also included. Typical rates of inactivation for the two Formalin concentrations are shown in Table 3. With 0.05% Formalin, inactivation was complete between 8 to 10 hr. Use of 0.1% Formalin decreased the inactivation period to between 6 to 8 hr.

Effect of Formalin concentration and length of inactivation period on vaccine potency. Thirty small lots of VEE vaccine were prepared by using either 0.05 or 0.1% Formalin with inactivation periods of 24 to 96 hr. Table 4 is a summary of the results of single-dose potency assays performed on these vaccines. These data indicate that VEE virus is stable antigenically, even after treatment with 0.1% Formalin for 96 hr at 37 C.

Selected lots of VEE vaccine were subjected to testing prior to final processing into multidose containers and were shown to be safe, sterile, and nontoxic.

DISCUSSION

The logistical advantages of the roller-bottle

TABLE 2. Effect of maintenance medium volume on propagation of VEE virus in chicken embryo cell roller-bottle cultures*

Time (hr) postinoculation	Log ₁₀ LD ₅₀ /ml by maintenance medium volume*		
	100 ml	200 ml	300 ml
2	3.2	3.3	3.0
6	6.5	6.3	6.0
12	9.9	9.0	9.0
18	9.9	10.3	9.9
24	10.0	9.3	9.3

* Multiplicity of inoculum, 0.006.

* Mean titers of samples obtained from replicate sets of cultures.

system in vaccine production were previously described (5). In the present study, we have used this technique to produce a potent, Formalin-inactivated VEE vaccine. As indicated by the results of studies in which various MOI were employed, great latitude is possible in selection of virus input. These results in concert with those obtained in the evaluation of the effect of MM volumes indicate that virus material of high titer could easily be obtained with a convenient 18 to 24 hr harvest time. Use of larger volumes of MM resulted in decreased cell destruction and therefore would insure minimal carry-over of cellular material into the final product. Thus, by careful selection of virus input and MM volume, one can adjust the system to comply with production

schedules as desired.

Our studies on Formalin inactivation at 37 C showed that although Trinidad strain VEE virus is resistant to heat inactivation, it is readily inactivated by both 0.05% and 0.1% Formalin at 37 C. Live virus was not detectable after 10 hr with 0.05% Formalin, or after 8 hr with 0.1%.

Recently, other authors (8, 15) noted the work of Smith, et al. (17) with regard to the inability to detect residual live virus in certain lots of VEE vaccines prepared from infected chicken embryos by Randall, Mills, and Engel (13), even though these same lots of vaccine had caused infection in 4% of 327 human vaccinees. Employing the best vaccine production techniques then available (13), these early workers attempted to inactivate the virus with 0.3% Formalin at 21 C for 4 days with periodic shaking of the vessel. The starting material was a crude 30% suspension of infected chicken embryos containing approximately 2 mg of protein N per ml. It is also significant that no filtration was employed to remove cellular debris. Furthermore, the authors described the final purified product, prepared by Sharples centrifugation as "...a pale yellow, slightly turbid fluid..." (13). All of these data strongly suggest that the virus was protected from the action of the Formalin as proposed by Smith et al. (17), perhaps by the location of the virus within cells or cellular debris. There was thus a possibility for the "unmasking" of the virus in man after vaccination.

In contrast, our method of inactivation consisted of the addition of 0.05 or 0.1% Formalin to a centrifuged and filtered, optically clear cell culture harvest that contained 0.29 to 0.38 mg of protein N per ml, the majority of which was the 0.25% human serum albumin added as a stabilizer (*unpublished data*). Thus, far less protein and little or no cellular debris were present to protect the virus. We inactivated at

TABLE 3. Formalin inactivation* of VEE virus at 37 C

Time (hr) after Formalin	Log ₁₀ LD ₅₀ /0.03 ml by Formalin concn of		Virus control ^b
	0.05%	0.1%	
0	8.7	8.7	8.7
1	5.0	4.5	
2	2.7	2.2	
3	1.5	<1.0	
4	1.0	<1.0	
5	<1.0	<1.0	
6	<1.0	<1.0	
8	<1.0	0 ^c	
10	0	0	
12	0	0	
14	0	0	
16	0	0	
24	0	0	7.2
96			1.5

* Determined by ic inoculation of 1- to 3-day-old mice with 0.03 ml.

^b Virus subjected to 37 C only.

^c Blanks, not tested.

^d Indicates survival of 50/50 mice inoculated with undiluted vaccine.

TABLE 4. Effect of Formalin concentration and length of inactivation period on potency of VEE vaccines

Formalin concn (%)	Median effective dose (ml) by hr of inactivation				
	24	30	48	72	96
0.05	0.032 ^a (0.026-0.042)	0.040 ^c (0.0072-0.11)	0.032	0.021	0.012 ^d (0.0041-0.019)
0.1	0.015	0.017 ^a (0.0039-0.030)	0.026	0.0081	0.014 ^c (0.0054-0.034)

^a Mean of three lots; values in parentheses indicate range of value for ED₅₀; when no range is shown, only one lot was tested.

^b Mean of 12 lots.

^c Mean of four lots.

^d Mean of two lots.

37 C with the vessels kept in constant motion throughout the periods employed. Other investigators have shown that temperature is critical and that lower temperatures greatly decrease the rate of inactivation (7, 16, 18).

In future studies, selected lots of inactivated Trinidad VEE vaccine will be tested in *Equidae* to establish parameters for volume and number of doses required, interval between doses, persistence of immunity, and effectiveness as a "booster" in equines previously immunized with attenuated VEE vaccine as well as in offspring of the latter.

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